Virology 275, 42–56 (2000)
doi:10.1006/viro.2000.0510, available online at http://www.idealibrary.com on IDEAL®

The Fitness of *Citrus Tristeza Virus* Defective RNAs Is Affected by the Lengths of Their 5′- and 3′-Termini and by the Coding Capacity

Munir Mawassi, Tatineni Satyanarayana, María R. Albiach-Martí, Siddarame Gowda, María A. Ayllón, Cecile Robertson, and William O. Dawson[1]

Department of Plant Pathology, University of Florida, Citrus Research and Education Center, Lake Alfred, Florida 33850

Received April 28, 2000; returned to author for revision June 20, 2000; accepted July 6, 2000

Populations of the *Closterovirus Citrus tristeza virus* (CTV) generally contain defective RNAs (dRNAs) that vary in size, abundance, and sequence. The variation in abundance of the different dRNAs in a population suggests selection for those of higher fitness. To examine factors affecting fitness of dRNAs, we investigated a series of *in vitro* constructed dRNAs for their ability to be amplified in protoplasts by an efficiently replicated CTV deletion mutant. The minimal sequences required for accumulation of the dRNAs were within the genomic 5′ proximal ~1 kb and the 3′ 270 nucleotides. However, other factors were involved, because a dRNA with only the minimal sequences failed to be replicated. Rescue of a nonviable dRNA by insertion of nonviral sequences between the termini suggested that "spacing" between terminal *cis*-acting signals influenced fitness. A continuous open reading frame (ORF) through most of the sequences derived from the 5′ of the genome was a requirement for dRNA amplification. In general, insertions, deletions, or nucleotide substitutions were tolerated in the dRNAs as long as an ORF was retained, whereas dRNAs with mutations that prematurely terminated the ORF were not viable. To discriminate between a requirement for an essential protein and ribosomal travel, perhaps to present replication signals to the replicase complex, mutations were made to modify the potential protein but still maintain an ORF. Deletions, insertions of nonviral sequences, or switching of reading frames that altered the amino acid sequence of the protein, except the N-terminal 161 amino acids, did not destroy the fitness of the dRNAs. Yet termination of the ORF in the middle of nonviral sequences did destroy the ability of the dRNAs to be amplified. These results suggest that even though a continuous ORF was needed for fitness, its protein product did not affect the amplification of the dRNAs.

INTRODUCTION

Citrus tristeza virus (CTV), the most destructive virus of the citrus industry worldwide (Bar-Joseph *et al.,* 1989), is a member of the *Closterovirus* genus within the *Closteroviridae.* The large, flexuous virion (2000 nm long) consists of a single-stranded positive-sense RNA genome of ~19.3 kb which is encapsidated by two coat proteins. The major coat protein (25 kDa) covers about 95% of the particle, while the minor coat protein (27 kDa) encapsidates the remaining terminus (Febres *et al.,* 1996). The genome consists of 5′- and 3′-nontranslated regions (NTRs) of 105–108 and 270 nucleotides (nts), respectively, and 12 open reading frames (ORFs) that potentially encode at least 19 protein products (Pappu *et al.,* 1994; Karasev *et al.,* 1995; Karasev, 2000). The 5′ ORF (ORF1a) encodes a large polyprotein with two papain-like protease domains, a methyltransferase-like, and a helicase-like domain. The putative RNA-dependent RNA polymerase is thought to be translated from ORF1b via a +1 frameshift. The ten 3′ ORFs are expressed through 3′ coterminal subgenomic RNAs (Hilf *et al.,* 1995).

A majority of CTV isolates are associated with multiple defective RNAs (dRNAs) that vary in size and abundance (Mawassi *et al.,* 1995a, b; Ayllón *et al.,* 1999). Some isolates have one or two dRNAs in major abundance along with multiple minor dRNAs. Most of the characterized dRNAs consist of simple fusions of the 5′- and the 3′-genomic termini, but their lengths and junction sites vary among dRNA species (Mawassi *et al.,* 1995b; Yang *et al.,* 1997b; Ayllón *et al.,* 1999). In contrast to defective interfering RNAs (DI RNAs) described for many animal and plant viruses (Holland, 1990; Roux *et al.,* 1991), CTV dRNAs do not noticeably interfere with virus accumulation (Mawassi *et al.,* 2000).

The genomic RNA of the virus contains, in addition to ORFs, sequences essential for gene regulation, replication, and genome packaging. dRNAs retain conserved *cis*-acting replication elements necessary for recognition by the replicase complex, but depend on the parental helper virus for the viral replicase and other *trans*-acting factors for replication. Some dRNAs associated with CTV consist of relatively small 5′- and 3′-termini, suggesting that the replication signals of CTV reside primarily in these terminal regions (Mawassi *et al.,* 1995b; Bar-Joseph *et al.,* 1997).

In addition to replicase recognition signals, other factors have been shown to influence dRNA fitness. One

[1] To whom reprint requests should be addressed at CREC, 700 Experiment Station Road, Lake Alfred, FL 33850. Fax: (863) 956-4631. E-mail: wodtmv@lal.ufl.edu.

0042-6822/00 $35.00

factor is the size of the dRNA molecule. For example, larger DI RNAs of *Broad bean mottle virus* (BBMV, Pogany *et al.,* 1997) and *Brome mosaic virus* RNA2 (Marsh *et al.,* 1991) accumulate to higher levels than smaller molecules. Yet, the opposite was found for DI RNAs of Sendai virus ranging from 1600 to 3500 nts (Re and Kingsbury, 1988). Also, studies on *Turnip crinkle virus* (TCV) DI RNAs have shown that the size of the molecule, rather than the specific sequence, affects the level of their accumulation (Zhang and Simon, 1994). In contrast, the fitness of *Tobacco mosaic virus* (TMV) dRNAs was not strictly correlated with size (Lewandowski and Dawson, 1998), suggesting that the juxtaposition of structure affected the ability to be replicated. The sizes of the CTV dRNA species range from small (~1.6 kb) to nearly genomic full-length (Bar-Joseph *et al.,* 1997; Yang *et al.,* 1997b; Mawassi *et al.,* 2000). The effects of the overall size and the lengths of termini on fitness of CTV dRNAs are not known.

Translation of an ORF through much of the dRNA can be another factor involved in efficient amplification of dRNAs. This could be due to either the production of a necessary *cis*-acting protein or the translation process itself (Jacobson and Peltz, 1996). With *Clover yellow mosaic virus* (CYMV) dRNAs, translatability rather than functional protein was suggested to be necessary for efficient accumulation *in vivo* (White *et al.,* 1992). In contrast, accumulation of some TMV dRNAs was facilitated by expression of a functional *cis*-encoded viral protein (Lewandowski and Dawson, 2000). However, some DI RNAs have limited (BBMV, Pogany *et al.,* 1997) or no (*Tomato bushy stunt virus;* Knorr *et al.,* 1991) coding capacity, demonstrating that this is not a general requirement. Sequence analysis of naturally occurring CTV dRNAs demonstrated that most had a large 5′ ORF (Mawassi *et al.,* 1995a, b; Yang *et al.,* 1997a, b), yet others did not contain a significant 5′ ORF (Ayllón *et al.,* 1999).

Previously, we developed a rapid and simple assay system to examine replication of *in vitro* constructed dRNAs in *Nicotiana benthamiana* protoplasts (Mawassi *et al.,* 2000). In this paper, we report the fitness of dRNAs with different combinations of 5′- and 3′-terminal sequences (the term terminal sequence refers to the sequences from one end of the RNA to the junction site) and with noncontiguous deletions or insertions of nonviral sequences. The results indicate that the sequences essential for minimal dRNA replication reside within the 5′ 1 kb and the 3′ NTR (270 nts) of the dRNA sequence, although internal sequences also affect dRNA accumulation. Our results also indicate that translation of the ORF in the 5′-terminal sequence of the dRNA appears to be required for efficient dRNA accumulation.

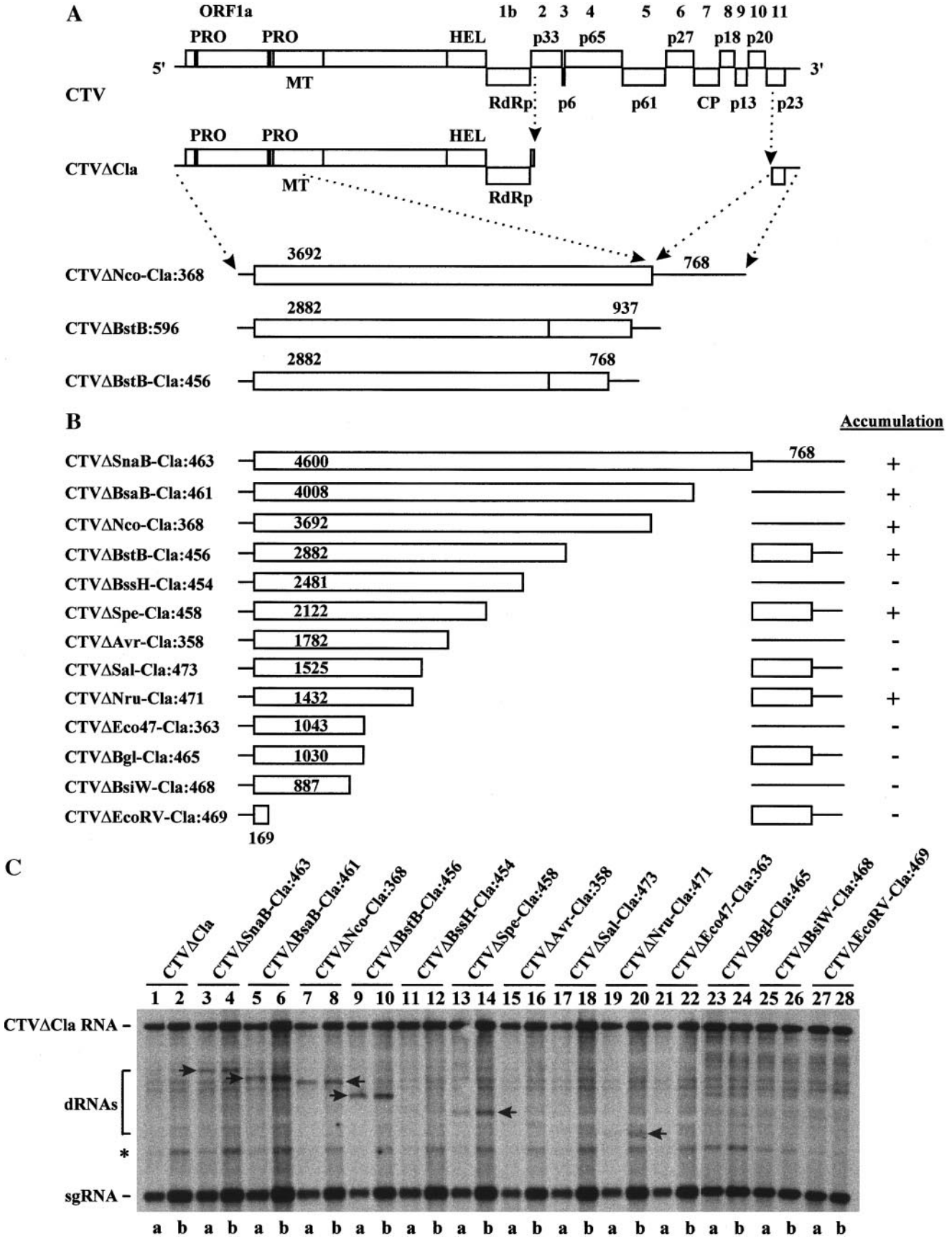
RESULTS

Effect of 5′-terminal sequences on the accumulation of the dRNAs

Previously, we created three biologically active dRNAs that contained *cis*-acting sequences essential for their replication *in trans* (Mawassi *et al.,* 2000). The 5′-terminal sequences of these dRNAs (CTVΔBstB-Cla:456, CTVΔBstB:596, and CTVΔNco-Cla:368) consist of 2882 or 3692 nts from the 5′-terminus of the CTV-T36 genomic RNA joined to the 3′-terminal 768 or 937 nts (Fig. 1A). Since some naturally occurring CTV dRNAs contain fewer 5′- or 3′-terminal nucleotides (Mawassi *et al.,* 1995a, b; Bar-Joseph *et al.,* 1997), we reasoned that some sequences within the *in vitro* synthesized dRNAs (Mawassi *et al.,* 2000) were probably in excess of the minimal replication signals and therefore could be deleted.

To examine the minimal 5′-terminal sequence that could support dRNA replication, we constructed a nested set of dRNAs with 5′-termini between 169 and 4600 nts, joined to a constant 3′-terminus of 768 nts (Fig. 1B). *In vitro* transcripts of the resulting cDNAs were examined for their ability to be replicated in *N. benthamiana* protoplasts using CTV-ΔCla as helper virus (Satyanarayana *et al.,* 1999). CTV-ΔCla contains the 5′ NTR plus ORFs 1a and 1b and the first 100 nts of ORF2 (p33) (nts 1–11011) joined to the 3′ 768 nts (nts 18528–19296) of the genomic RNA, which includes most of ORF11 (p23) and the 3′ NTR (Fig. 1A). Total RNA was extracted from the transfected protoplasts at 3 and 4 days postinoculation (p.i.), a time when dRNAs approached maximal accumulation levels (Mawassi *et al.,* 2000), and was analyzed by Northern blot hybridizations using T36 3′ positive- and negative-stranded RNA-specific probes. However, because the input RNA transcripts that persisted in the cells obscured the detection of accumulation of progeny positive-stranded RNA (Mawassi *et al.,* 2000), we present hybridizations with the negative-stranded RNA-specific probe (Fig. 1C). Analysis of protoplasts inoculated with only RNA transcripts from the helper, CTV-ΔCla, revealed two RNAs corresponding to the genomic RNA (~12.0 kb) and a subgenomic RNA initiated by the p33 subgenomic RNA promoter (~0.85 kb) (Fig. 1C, lanes 1 and 2).

Among the dRNAs with different lengths of 5′ sequences, those with 5′-terminal sequences of 2882 nts and larger accumulated to detectable levels (Fig. 1C, lanes 3–10). Replication of the dRNA with a 5′-terminus of 2481 nts was not detected (Fig. 1C, lanes 11 and 12), but that with 2122 nts was detected (Fig. 1C, lanes 13 and 14). Further reductions in the size of the 5′-terminus to 1782 or 1525 nts did not result in dRNA accumulation (Fig. 1C, lanes 15–18), but the dRNA with a 5′-terminal sequence of 1432 nts was replicated weakly (Fig. 1C,


A
ORF1a
1b 2 3 4 5 6 7 8 9 10 11
PRO PRO HEL p33 p65 p27 p18 p20
5' 3'
MT
CTV
RdRp p6 p61 CP p13 p23
PRO PRO HEL
CTVΔCla
MT
RdRp
3692
768
CTVΔNco-Cla:368
2882
937
CTVΔBstB:596
2882
768
CTVΔBstB-Cla:456
B
Accumulation
CTVΔSnaB-Cla:463 4600 768 +
CTVΔBsaB-Cla:461 4008 +
CTVΔNco-Cla:368 3692 +
CTVΔBstB-Cla:456 2882 +
CTVΔBssH-Cla:454 2481 -
CTVΔSpe-Cla:458 2122 +
CTVΔAvr-Cla:358 1782 -
CTVΔSal-Cla:473 1525 -
CTVΔNru-Cla:471 1432 +
CTVΔEco47-Cla:363 1043 -
CTVΔBgl-Cla:465 1030 -
CTVΔBsiW-Cla:468 887 -
CTVΔEcoRV-Cla:469 169 -
C
CTVΔCla
CTVΔSnaB-Cla:463
CTVΔBsaB-Cla:461
CTVΔNco-Cla:368
CTVΔBstB-Cla:456
CTVΔBssH-Cla:454
CTVΔSpe-Cla:458
CTVΔAvr-Cla:358
CTVΔSal-Cla:473
CTVΔNru-Cla:471
CTVΔEco47-Cla:363
CTVΔBgl-Cla:465
CTVΔBsiW-Cla:468
CTVΔEcoRV-Cla:469
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
CTVΔCla RNA –
dRNAs
*
sgRNA –
a b a b a b a b a b a b a b a b a b a b a b a b a b a b a b a b

lanes 19 and 20). None of the dRNAs with a 5′ sequence less than 1432 nts accumulated to detectable levels (Fig. 1C, lanes 21–28). These results suggested that the minimal 5′ replication signals of CTV dRNAs resided within the 5′ 1432 nts (dRNA CTVΔNru-Cla:471). However, the fact that some of the dRNAs with 5′-terminal sequences larger than 1432 nts were unable to accumulate to detectable levels suggested that a factor(s) other than the length of the 5′-terminal sequence affected the replication efficiency.

In another approach to examine requirements for dRNA accumulation, we introduced noncontiguous internal deletions into the 5′-terminal sequence (3692 nts) of the dRNA CTVΔNco-Cla:368. Internal sequences ranging in size from 70 to 538 nts were deleted from different regions of the 5′-terminal sequence, thus creating dRNAs with noncontiguous segments (Fig. 2A). These dRNAs were coinoculated into protoplasts with CTV-ΔCla as the helper virus, and dRNA accumulation was examined by Northern blot hybridization analysis. Among these dRNAs, only CTVΔNco-ClaΔNsi:381 accumulated to detectable levels, indicating that the dRNA CTVΔNco-Cla:368 was capable of tolerating an additional internal deletion. Although this deletion prematurely terminated the ORF, the 5′-terminal sequence of the resultant dRNA still had an ORF constituting ~75% of the dRNA sequence. The other deletions abolished replication (Fig. 2A). Among these, with the exception of CTVΔNco-ClaΔBsiW:397, the deletions truncated the 5′ ORF of the dRNAs.

Because the lack of detectable accumulation of the above dRNAs could have resulted from factors other than 5′ replication signals, such as translation of a relatively large ORF, we introduced internal deletions within the smaller dRNA, CTVΔBstB:596, that were designed to retain a continuous ORF (Fig. 2B). The deletions ranged in size from 59 to 421 nts. Except for dRNA CTVΔBstBΔ0.88–1.02:788, these dRNAs were replicated. These results suggested that: (i) the deleted sequences were not required for replication; (ii) the 5′ 1032 nts were sufficient for the dRNAs to be replicated (CTVΔBstBΔ1.03–1.09:752 and CTVΔBstBΔ1.03–1.45:675); and (iii) CTV dRNAs with three noncontiguous segments were capable of being replicated *in trans*.

Relationship between the length of the 3′-terminus and dRNA accumulation

We next examined 3′ sequence requirements by making a nested set of deletions resulting in dRNAs with different sizes of 3′ sequences, ranging from 170 to 1038 nts joined to a constant 5′-terminal sequence of 2882 nts (Fig. 3A).

The dRNAs that retained the 3′-terminal NTR (270 nts) or a larger sequence accumulated to detectable levels (Fig. 3B, lanes 3–12). We did not detect replication of the dRNAs that had a 3′-terminal sequence of 170 or 220 nts (Fig. 3B, lanes 13–16), suggesting that the 3′ signals essential for replication *in trans* resided within the 3′ NTR. Figure 3B also shows that the accumulation levels varied between the dRNAs. Under our conditions, optimal accumulation was obtained for the dRNA CTVΔBstB:596 that retained the 3′-terminal 937 nts.

Replication of dRNAs with minimal 5′- and 3′-termini

The minimal 5′- and 3′-termini determined above functioned on dRNAs that contained greater than minimal opposite termini. To examine whether a dRNA containing minimal terminal sequences at both ends could replicate, a dRNA with a 5′-terminus of 1032 nts and a 3′-terminus of 270 nts (3′ NTR) was created. After transfection of protoplasts with this dRNA and helper RNA, there was no evidence of dRNA accumulation (results not shown).

Examination of ORFs and dRNA fitness

In the earlier experiments, dRNAs with longer 5′ ORFs tended to be replicated more efficiently (Fig. 1C, lanes 3–8; Fig. 2), suggesting that replication of these dRNAs was facilitated due to a large ORF within their 5′ regions. However, the fact that some smaller dRNAs were also replicated suggested that translation of a contiguous hybrid ORF extending through the ligation junction also enabled replication.

The sequences of the dRNAs CTVΔBstB:596 and CTVΔBstB-Cla:456 contained ORFs that continued through the junction sites (Fig. 1A). *In vitro* translation analysis in rabbit reticulocyte lysate confirmed that these dRNAs contained continuing ORFs (results not shown).

FIG. 1. Effect of size of the 5′- terminal sequence on dRNA accumulation. (A) Schematic diagrams of the genomic organization of CTV, the deletion mutant CTV-ΔCla, and the synthetic dRNAs CTVΔNco-Cla:368, CTVΔBstB:596, and CTVΔBstB-Cla:456. Translation products encoded by the open reading frames (ORFs, numbered above the CTV diagram) are indicated. The putative domains PRO, MT, HEL, and RdRp represent papain-like proteases, methyltransferase, helicase, and RNA-dependent RNA polymerase, respectively. CP, the 25-kDa coat protein ORF. (B) dRNA-like constructs having different 5′-termini and a constant 3′-terminus. Boxes and solid lines represent translatable and nontranslatable sequences, respectively; gaps represent deleted sequences. The lengths of the terminal sequences (from the terminus to the junction) are indicated. The replicability of the dRNAs in *Nicotiana benthamiana* protoplasts is indicated to the right. (C) Northern blot analysis showing the accumulation levels of the synthetic dRNAs in protoplasts, at 3 (lanes a) and 4 (lanes b) days postinoculation (p.i.), using CTV-ΔCla as helper virus. The hybridization was carried out with a 3′ negative-stranded RNA-specific probe. Arrows indicate the positions of the dRNAs. The positions of CTV-ΔCla RNA and the subgenomic RNA are indicated to the left. The asterisk indicates the position of a nonspecific band.

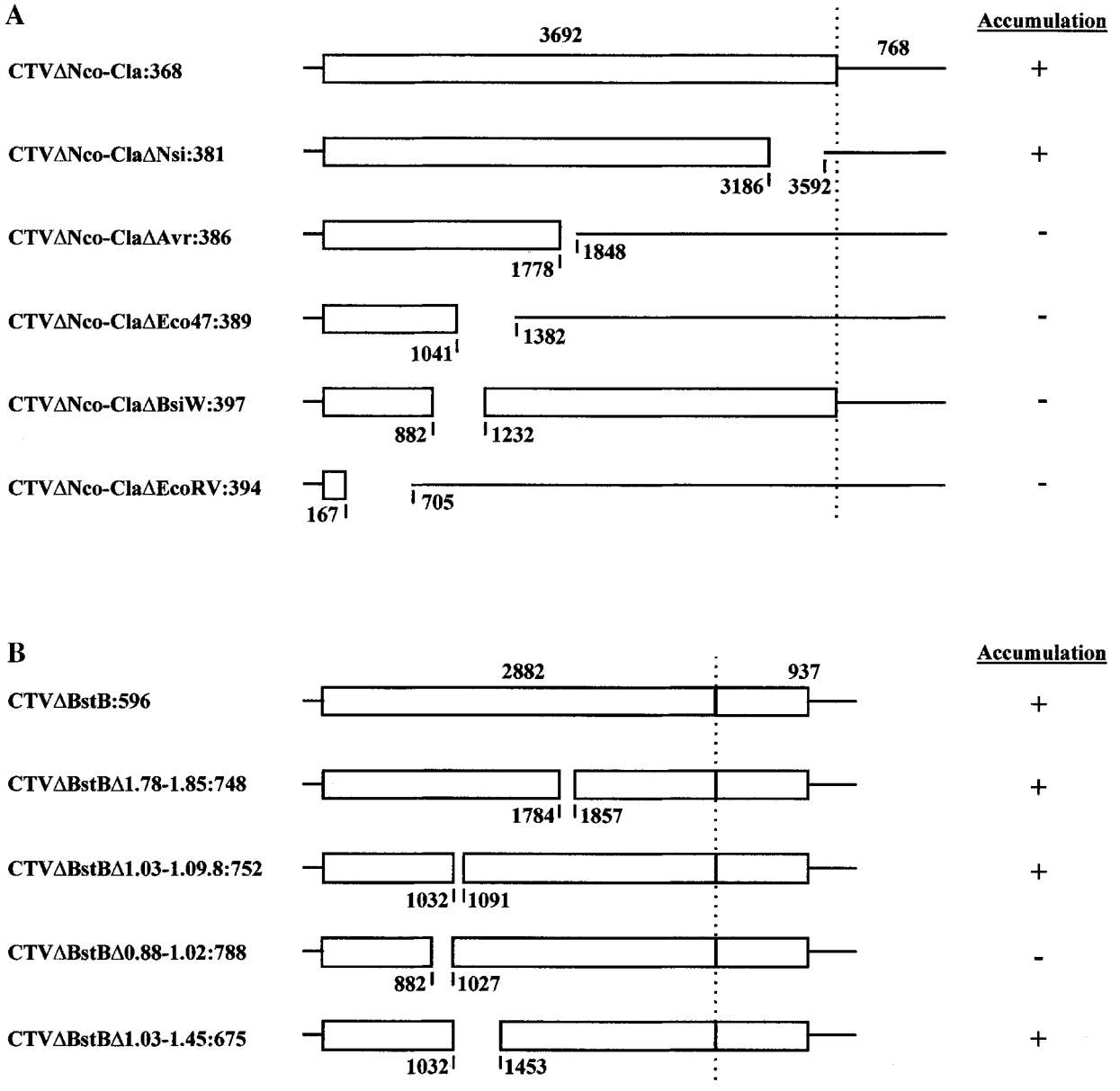


FIG. 2. Effect of noncontiguous internal deletions on dRNA accumulation. Schematic diagrams of dRNA derivatives of CTVΔNco-Cla:368 (A) and CTVΔBstB:596 (B) with internal deletions. Boxes and solid lines represent translatable and nontranslatable sequences, respectively; gaps represent deleted sequences; vertical dotted lines depict the position of the junctions. The lengths of the termini and the positions of the deletions are indicated. The accumulation of each dRNA in protoplasts is indicated.

To examine whether replication of these dRNAs *in trans* required translation through the junction, the ORFs were truncated at the ligation junction sites by the insertion of stop codons. A single point mutation $U_{2898} \rightarrow G$ was introduced in CTVΔBstB:596 to create a stop codon (UAG) four amino acids downstream of the junction site (Fig. 4A). In CTVΔBstB-Cla:456, two extra bases were introduced at the junction site to terminate the reading frame, two amino acids downstream of the junction (Fig. 4A). Both resulting dRNAs were efficiently amplified (Fig. 4B), demonstrating that translation through the splice junction was not required for replication of these dRNAs.

We next examined whether premature termination of translation within the sequences 5′ of the junction affected dRNA propagation. The ORF of the dRNA CTVΔBstB-Cla:456 was disrupted by insertion of four additional nts, resulting in a frameshift and premature termination of the predicted translation product. Frameshift mutations were introduced into the dRNA sequence at position 1027, 1457, or 2482 to truncate the predicted protein product after 352, 514, or 858 amino acid residues, respectively. None of the frameshift derivatives accumulated to detectable levels (results not shown).

In these frameshift mutants, translation terminated in the new reading frame, several amino acid residues downstream of the mutation sites, resulting in the addi-

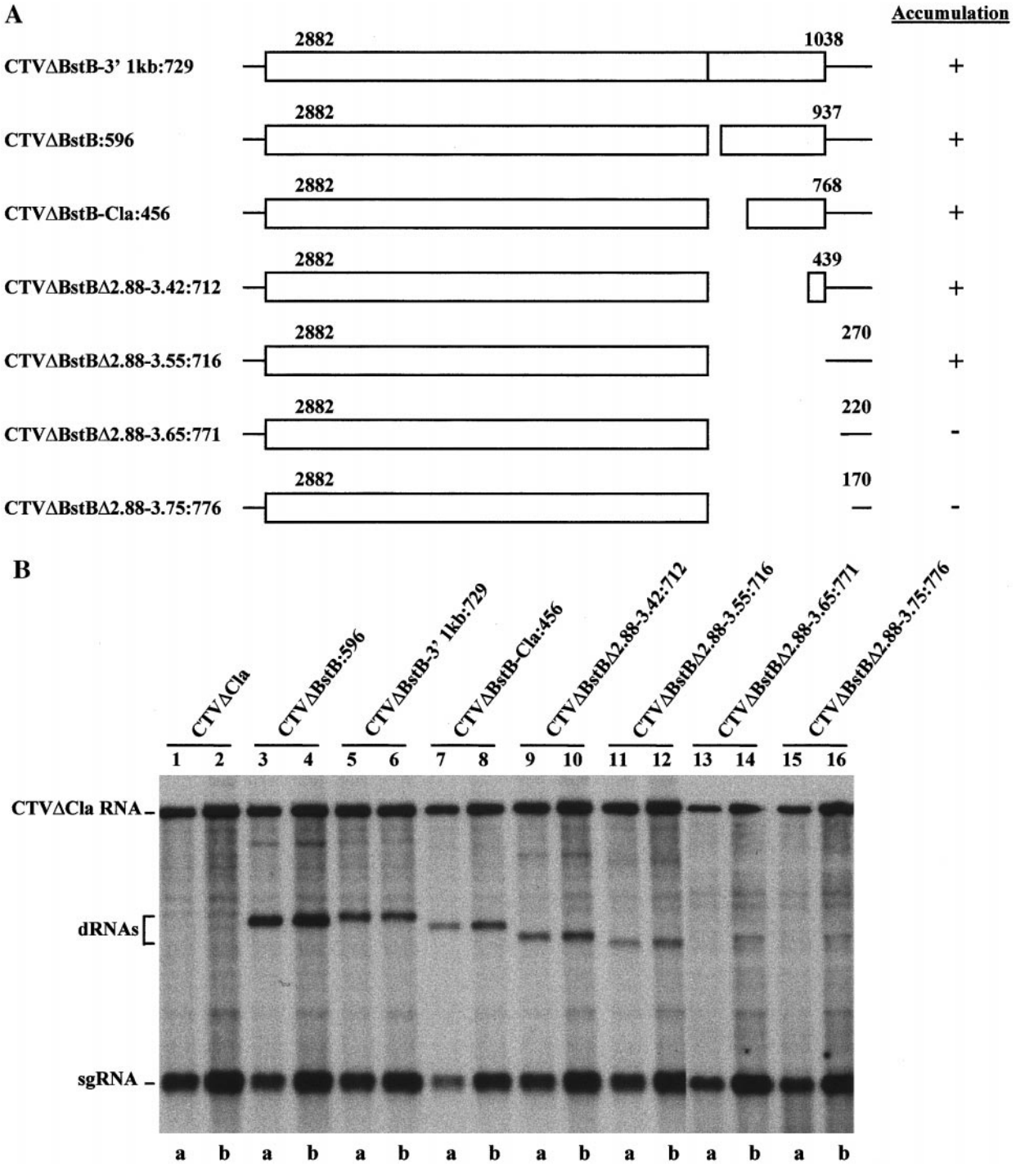


FIG. 3. Replication of synthetic dRNAs having different 3′-termini and a constant 5′-terminus. (A) Schematic diagrams of the dRNA constructs. Boxes and solid lines represent translatable and nontranslatable sequences, respectively; gaps represent deleted sequences. The positions and lengths of the termini are indicated. The replicability of the dRNAs is indicated to the right. (B) Analysis of dRNA accumulation in protoplasts at 3 (lanes a) and 4 (lanes b) days p.i. using a 3′ negative-stranded RNA-specific probe. The positions of the helper CTV-ΔCla RNA, the subgenomic RNA, and the dRNAs are indicated.

tion of nonviral-encoded amino acid residues. To examine whether the premature termination of the ORF or the presence of the additional amino acid residues affected the dRNA accumulation, we disrupted the ORF by directly inserting a stop codon. The UAG stop codon was inserted in the reading frame of CTVΔBstB-Cla:456 at position 1036 (Fig. 5A). Protoplasts inoculated with the resulting dRNA and helper RNA did not accumulate progeny dRNA (Fig. 5B, lanes 3 and 4). To ensure that the replication was affected by the stop codon (UAG) and not

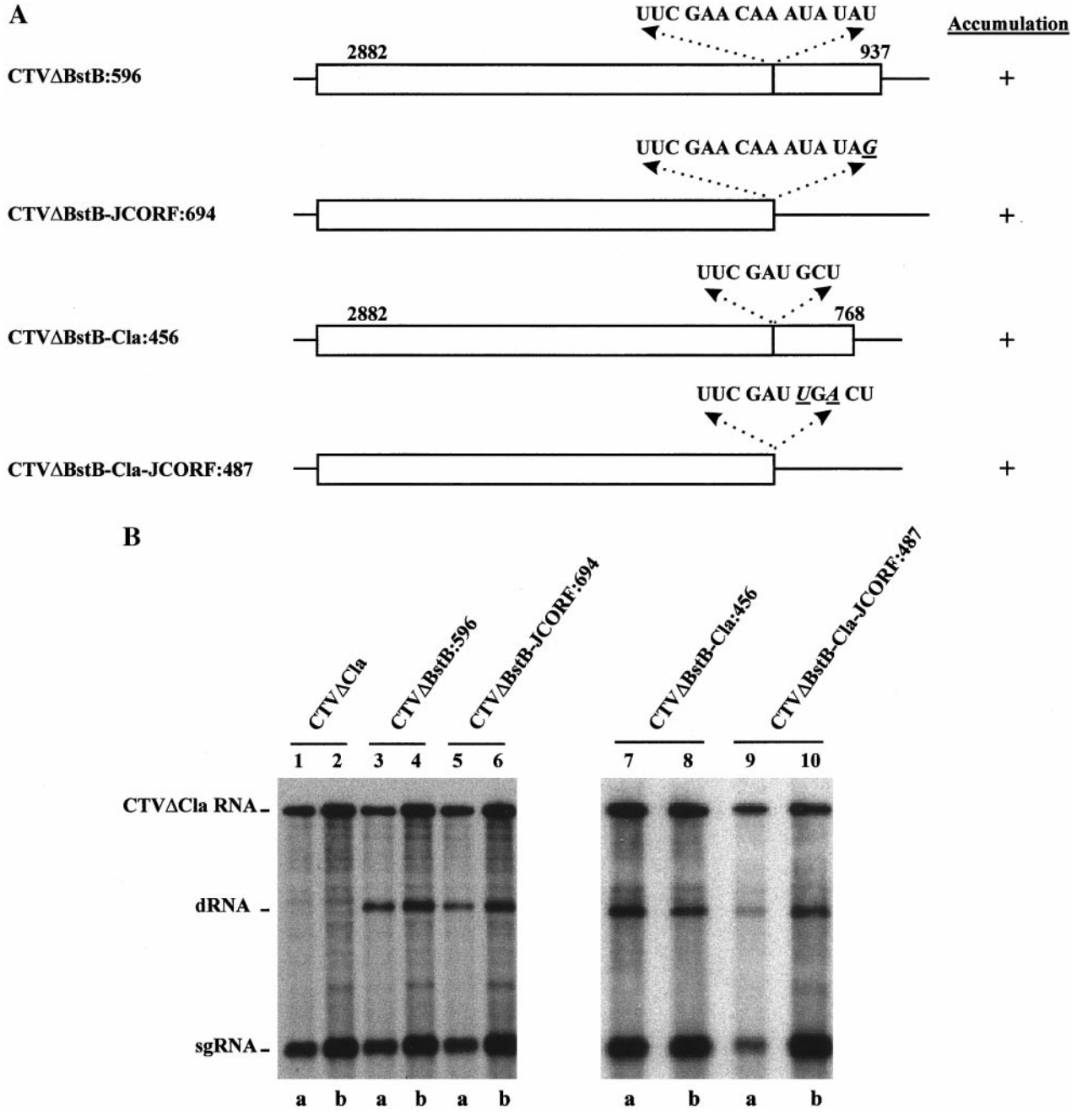


FIG. 4. Replication of synthetic dRNAs containing ORFs truncated at the ligation junction sites. (A) Schematic diagrams of the dRNAs and the nucleotide sequences at the junctions. The underlined and italicized nucleotides were introduced to create stop codons. Boxes and solid lines represent translatable and nontranslatable sequences, respectively. The lengths of the termini are indicated. The replicability of the dRNAs is indicated to the right. (B) Northern blot hybridizations showing the accumulation levels in protoplasts at 3 (lanes a) and 4 (lanes b) days p.i. using a 3′ negative-stranded RNA-specific probe. The positions of the helper CTV-ΔCla RNA, the subgenomic RNA, and the dRNAs are indicated.

by the insertion of 3 nts, a CAC codon encoding His was inserted at position 1036 (Fig. 5A). The resulting dRNA was replicated efficiently (Fig. 5B, lanes 5 and 6).

In another approach to terminate the ORF without altering the overall size of the dRNA, we used a nucleotide substitution instead of insertion (Fig. 5A). Replacement of the GAU Asp codon, at position 1657 in CTVΔBstB-Cla:456, with the UAG stop codon was lethal (Fig. 5B, lanes 7 and 8), while replacement of the same GAU codon with GAG encoding Glu resulted in replication (Fig. 5B, lanes 9 and 10). Similarly, we did not detect accumulation of dRNA that had the CCG Pro codon at position 268 replaced with UAG (results not shown). However, the accumulation levels were not affected when the same CCG Pro codon was substituted with AAG encoding Lys (results not shown). The single point

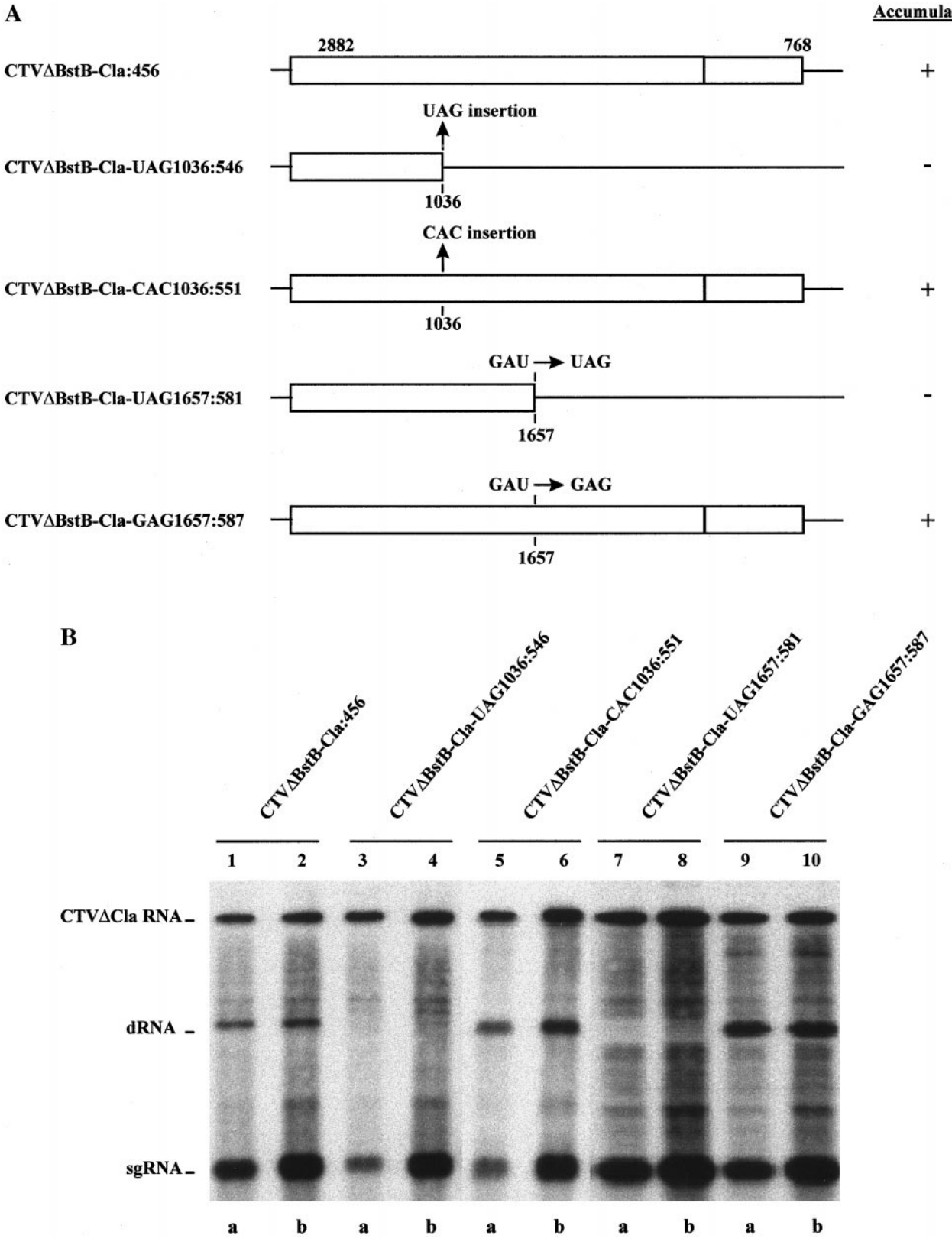


FIG. 5. Effect of prematurely terminated ORFs on accumulation of CTV dRNAs. (A) Schematic diagrams of dRNAs with insertion or substitution of nucleotides which were introduced to create stop codons or introduce different amino acid residues. Boxes and solid lines represent translatable and nontranslatable sequences, respectively. The lengths of the termini are indicated. The replicability of the dRNAs is indicated to the right. (B) Northern blot hybridizations showing the accumulation levels in protoplasts at 3 (lanes a) and 4 (lanes b) days p.i. using a 3′ negative-stranded RNA-specific probe. The positions of the helper CTV-ΔCla RNA, the subgenomic RNA, and the dRNAs are indicated.

mutations $C_{173} \rightarrow A$ or $U_{2487} \rightarrow G$ that truncated the predicted translation product at amino acid residues 57 and 828, respectively, did not result in detectable dRNA amplification (results not shown). The effects of premature termination of translation on accumulation of the dRNA CTVΔBstB:596 (results not shown) were similar to those shown for CTVΔBstB-Cla:456.

These results suggested that premature termination of the ORF within the sequences from the 5′-terminus negatively affected fitness of dRNAs. Also, the results demonstrated that small in-frame insertions and nucleotide substitutions could be tolerated if the continuous ORF was maintained within the 5′-terminal sequence of the dRNA.

Effect of translation or *cis*-acting protein on accumulation of the dRNA

Retaining a continuous ORF through the 5′-terminal sequence of the dRNA CTVΔBstB-Cla:456 or CTVΔBstB:596 apparently was needed for the dRNA to be replicated *in trans.* However, these data do not differentiate whether the translated protein was involved in dRNA replication or whether the translation process, i.e., movement of the ribosome on the mRNA, was needed.

One approach to examine whether a dRNA protein is involved in dRNA replication is to determine whether supplying the predicted protein *in trans* from a replicating dRNA would rescue a nonviable dRNA. We examined the amplification of the dRNA CTVΔSal-Cla:473, which had one ORF continuing across the junction, but failed to accumulate to detectable levels (Fig. 1C, lanes 17 and 18), by coinoculation with the efficiently replicating dRNA, CTVΔBstB:596, and the helper, CTV-ΔCla. The helper and CTVΔBstB:596 were amplified, but CTVΔSal-Cla:473 was not (results not shown). Replication of CTVΔBstB:596 and its putative protein product did not rescue CTVΔSal-Cla:473 *in trans.* However, these results did not exclude the possibility that *in trans* replication of CTV dRNA requires production of a *cis*-acting protein.

To further examine whether dRNA replication required a protein acting *in cis,* we mutated the ORF of CTVΔBstB:596. First, a large fragment of the predicted protein product was deleted. The in-frame deletion of the sequence 1033 to 2882 (junction site) resulted in the dRNA CTVΔBgl-BstB:691 with 1032 and 937 nts of the 5′- and 3′-termini, respectively (Fig. 6A). Even though these termini included 5′ and 3′ sequences found to be necessary for replication *in trans,* this dRNA failed to be replicated (Fig. 6B, lanes 1 and 2). Next, the green fluorescent protein (GFP) ORF (lacking a stop codon) was inserted in-frame, replacing CTV sequences previously deleted in CTVΔBgl-BstB:691 (Fig. 6A). The resultant dRNA was replicated efficiently (Fig. 6B, lanes 3 and 4), suggesting that the predicted peptide product encoded by sequences downstream of nucleotide 1032 was not essential for replication of the dRNA.

This experiment did not exclude the possibility that the predicted protein, which is encoded from the 5′ 1 kb of the dRNA sequence, was required for replication. Introducing deletions within this region would likely interfere with the 5′ replication signals to the detriment of dRNA propagation. Therefore, to examine this possibility we introduced mutations within this 5′ 1-kb sequence that would mutate the predicted encoded product, but minimally disturb the 5′ replication signals (Fig. 7A). Two extra nts were inserted at position 596 in CTVΔBstB:596 to cause a frameshift within the ORF (dRNA CTVΔBstB-FS1:769). The new ORF terminated at nucleotide 840. There was no detectable accumulation of this frameshift mutant (Fig. 7B, lanes 3 and 4). Further deletion of a single nucleotide (U at position 840) resulted in an ORF shift and an extended new ORF that terminated at nucleotide 969 (dRNA CTVΔBstB-FS2:791). Again, this dRNA was not replicated (Fig. 7B, lanes 5 and 6). Next, we deleted the nucleotide U at position 970, causing the new ORF to be shifted to rejoin the original ORF of CTVΔBstB:596. The resultant frameshift derivative, CTVΔBstB-FS3:792, had a similar size and nearly an identical nucleotide sequence as CTVΔBstB:596, but encoded a predicted translation product containing the substitution of 124 amino acid residues. This dRNA was accumulated efficiently in protoplasts (Fig. 7B, lanes 7 and 8), indicating that a large part (amino acids 162–286) of the predicted translation product encoded by the 5′-terminal sequence of the dRNA CTVΔBstB:596 was not required for its replication. Moreover, these results suggested that the ORF messenger activity was an essential element for replication of CTV dRNAs.

To further examine whether ribosomal travel or *cis*-acting protein was important for dRNA propagation, we designed a dRNA which had an insertion of a nonviral sequence within the ORF. If the ability of the dRNA to be replicated is dependent upon translation of the nonviral sequence, then it is likely that the coding capacity, and not the specific sequence of the encoded translation product, is required for replication *in trans.*

For this purpose we used the dRNA CTVΔBstBΔ1.03–1.45:675, a derivative of CTVΔBstB:596 (with the deletion 1033–1452), which accumulated in protoplasts efficiently (Figs. 2B and 8). The GFP ORF, lacking a stop codon, was fused in-frame to the dRNA sequence to replace the deleted sequence (Fig. 8A). Protoplasts inoculated with the resultant dRNA (CTVΔBstBΔ1.03–1.45+GFP:754) and CTV-ΔCla, as helper virus, resulted in accumulation of dRNA progeny (Fig. 8B, lanes 3 and 4). However, the $U_{438} \rightarrow G$ point mutation that truncated the GFP ORF in the middle of its sequence resulted in loss of replicability of the dRNA (Fig. 8B, lanes 5 and 6). Likewise, replacement of a similar deletion of 1033–1452 in CTVΔBstB-Cla:456 sequence by an in-frame GFP ORF resulted in effi-

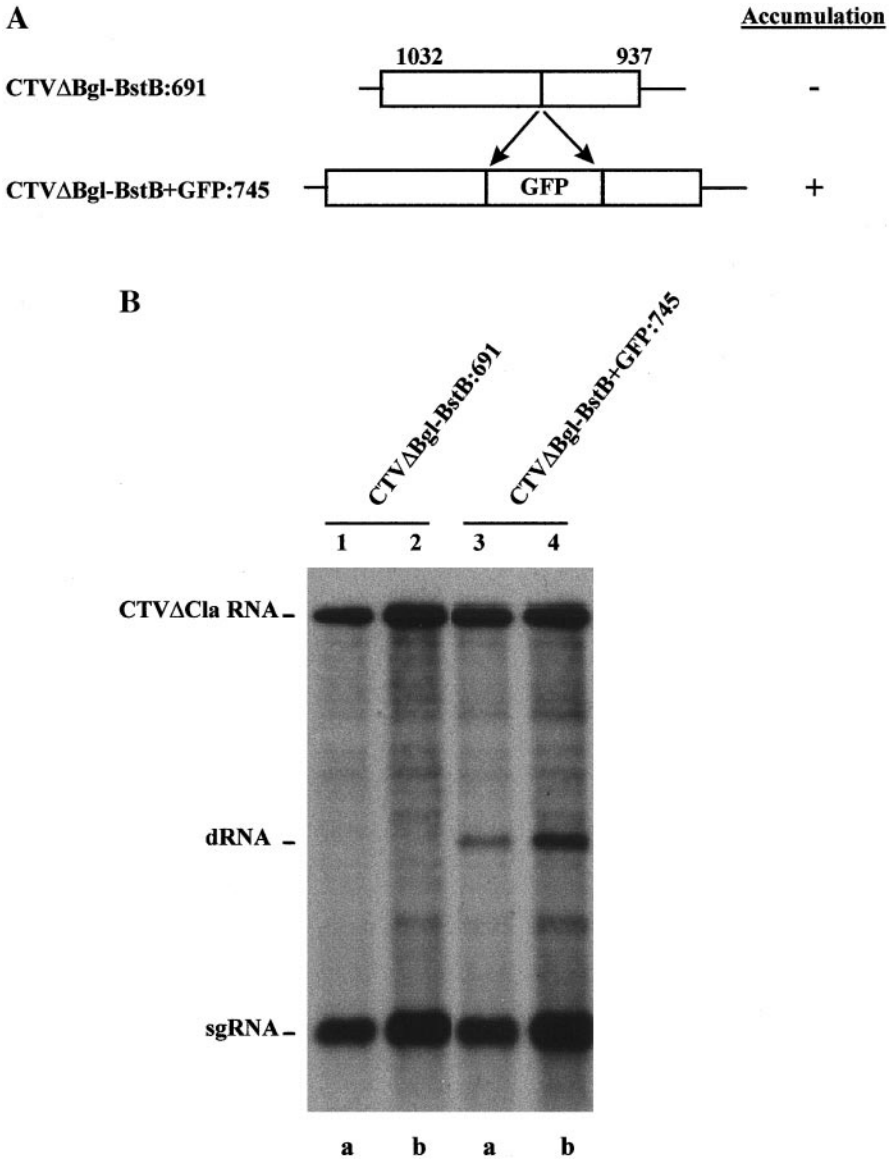


FIG. 6. Replication of CTV dRNAs with nonviral sequences. (A) Schematic diagrams of the dRNAs. The GFP ORF was inserted in-frame in the junction of CTVΔBgl-BstB:691. The lengths of the termini are indicated. The replicability of the dRNAs is indicated to the right. (B) Northern hybridization blot showing the accumulation levels of the dRNA progeny in protoplasts at 3 (lanes a) and 4 (lanes b) days p.i. using a 3′ negative-stranded RNA-specific probe. The positions of the helper CTV-ΔCla RNA, the subgenomic RNA, and the dRNA are indicated.

cient accumulation (Fig. 8B, lanes 7 and 8), whereas introducing the UAG stop codon within the nonviral GFP ORF prevented dRNA amplification (Fig. 8B, lanes 9 and 10). Thus, these results show that translation, even of the nonviral sequence, supported dRNA replication *in trans,* suggesting that the translation process, but not the encoded translation product, determined dRNA fitness.

DISCUSSION

If CTV dRNAs were generated from random or near random recombination, one would expect a continuous spectrum of sizes of dRNA molecules to be produced in each isolate. In general, CTV-infected plants contain one or a few dRNAs that accumulate to high levels, suggesting that there is selection of those with higher fitness. In this study, we found that at least three factors affected accumulation of CTV dRNAs: (i) more than minimal 5′- and 3′-termini, (ii) "spacing" of the terminal sequences, and (iii) translation of an ORF continuing through the 5′-terminal sequence. However, other factors, such as RNA folding, might be involved as well.

It has been generally thought that smaller dRNAs can replicate more efficiently than larger dRNAs (Holland *et al.,* 1980). However, there appears to be little correlation between the overall size of naturally occurring CTV dRNAs and their accumulation levels. Abundant dRNAs

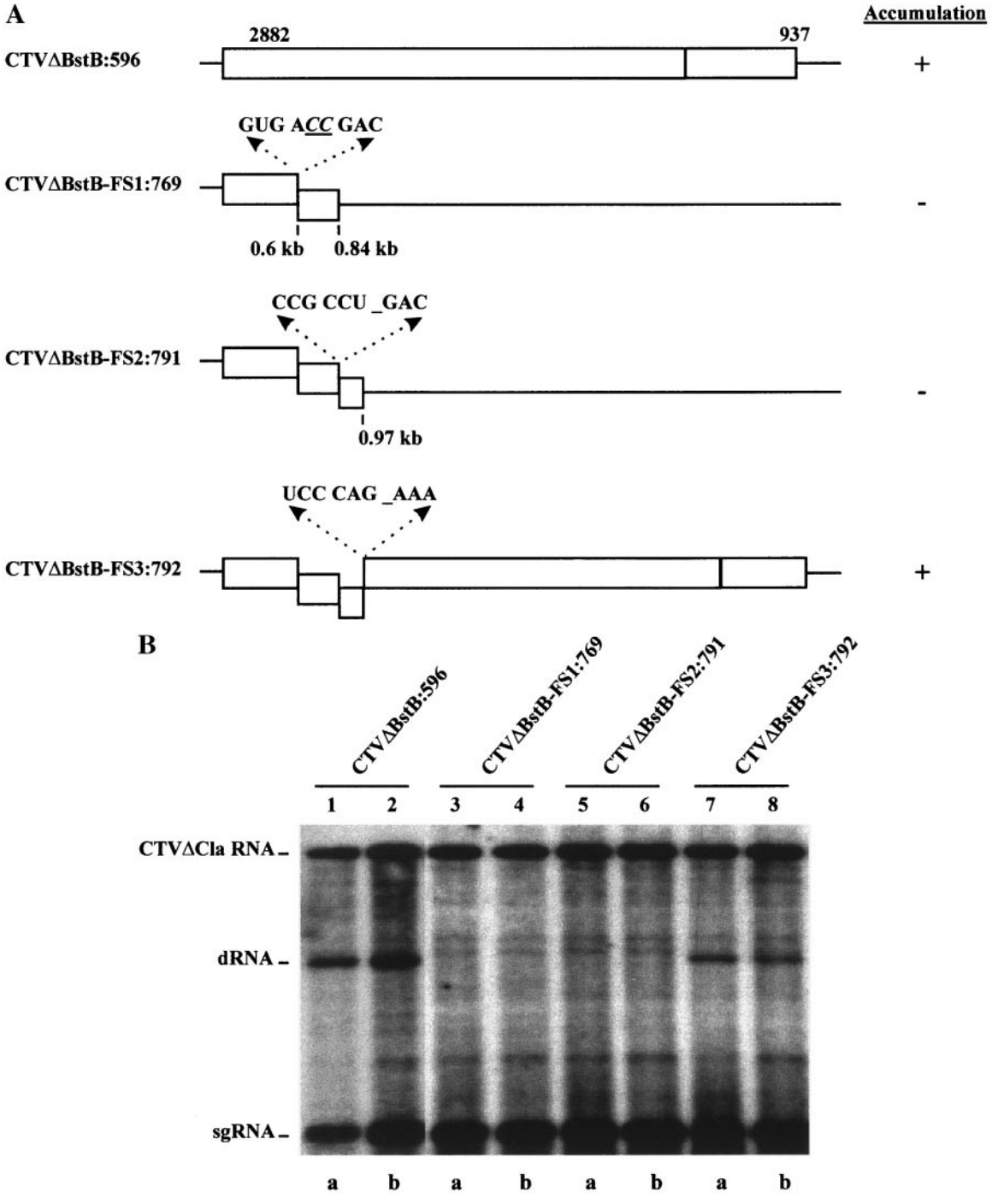


FIG. 7. Replication of CTV dRNAs containing frameshift mutations. (A) Schematic diagrams of the dRNAs. CTVΔBstB:596 was mutagenized by insertions (underlined and italicized) or deletions (_) of nucleotides to shift the translation of the ORF. Boxes and solid lines represent translatable and nontranslatable sequences, respectively. The replicability of the dRNAs is indicated to the right. (B) Northern blot hybridization showing the accumulation levels in protoplasts at 3 (lanes a) and 4 (lanes b) days p.i. using a 3′ negative-stranded RNA-specific probe. The positions of the helper CTV-ΔCla RNA, the subgenomic RNA, and the dRNAs are indicated.

vary from ~1.6 kb to more than 10 kb (Mawassi *et al.,* 2000). Among the smaller dRNAs that have been characterized, the size of the 5′-termini generally was larger than 1.1 kb and the 3′-termini vary from ~0.4 to 1.2 kb (Mawassi *et al.,* 1995b; Bar-Joseph *et al.,* 1997). Here, we examined replication of synthetic CTV dRNAs of different sizes and found that, although the accumulation levels were not correlated with the overall size, their ability to be replicated was affected by the lengths of the termini. Progressive deletions within these dRNAs indicated that the minimal terminal sequences required for replication *in trans* resided within the 5′ 1032 nts and the 3′ 270 nts

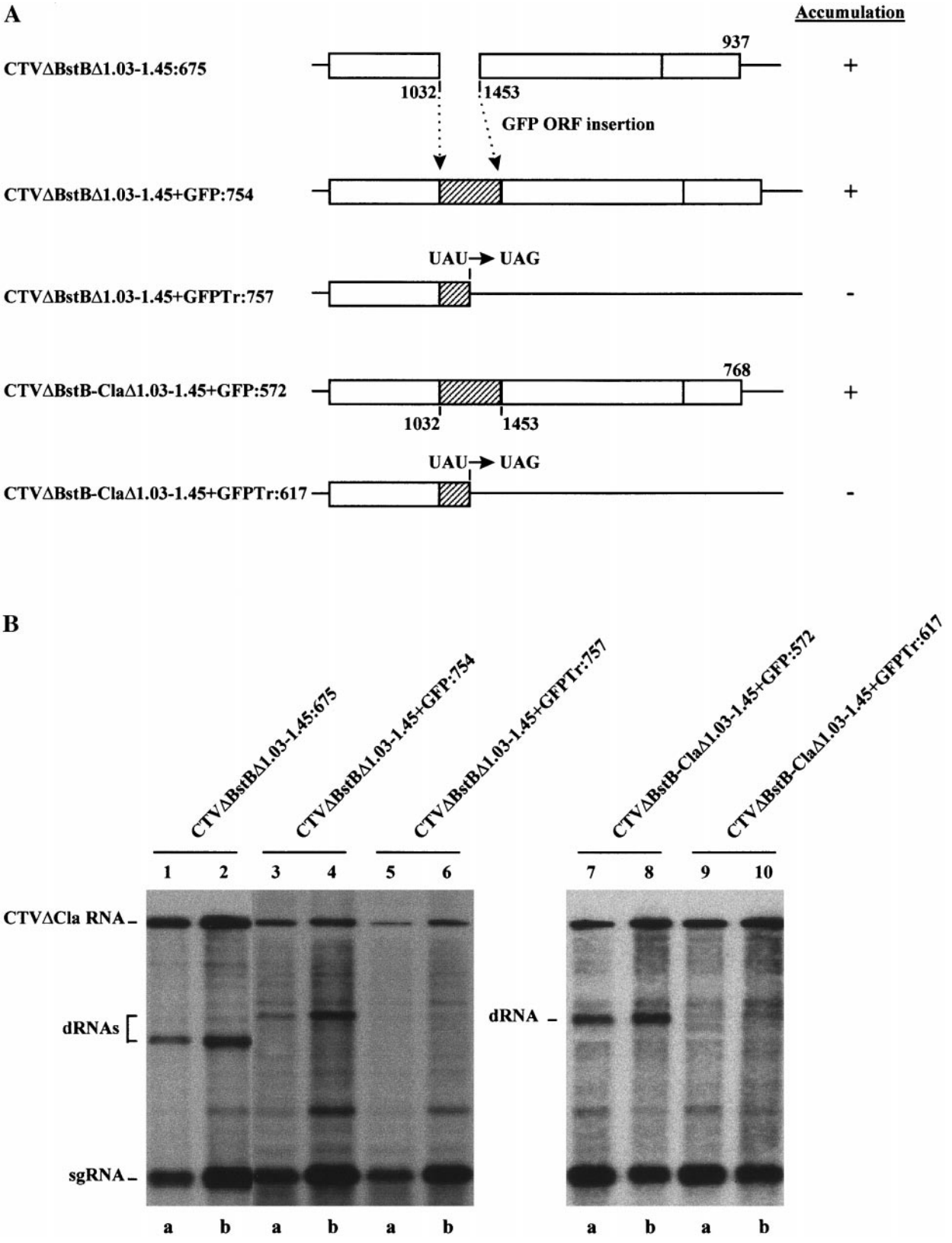


FIG. 8. Effect of translation on dRNA accumulation. (A) Schematic diagrams of the dRNAs. The CTV sequence 1033–1452 was replaced with the GFP ORF, and an amino acid residue in the middle of the nonviral sequence was mutated to create a termination codon; shaded boxes, GFP ORF; boxes, translatable sequences; lines, nontranslatable sequences. The replicability of the dRNAs is indicated to the right. (B) Northern blot hybridizations showing the accumulation levels in protoplasts at 3 (lanes a) and 4 (lanes b) days p.i. using a 3′ negative-stranded RNA-specific probe. The positions of the helper CTV-ΔCla RNA, the subgenomic RNA, and the dRNAs are indicated.

(3′ NTR). Interestingly, among the different 3′-termini, the highest accumulation levels were detected for the dRNA with the 3′-terminus of 937 nts (CTVΔBstB:596), suggesting that this size or sequence, which was found to be common for a large proportion of naturally occurring CTV dRNAs, might provide near optimal fitness. Minimal 5′- and 3′-termini alone, however, were insufficient for replication, suggesting that some internal sequences increased dRNA fitness.

The fitness of DI or dRNAs is often affected by internal contiguous (Pogany *et al.,* 1997) or noncontiguous (Knorr *et al.,* 1991; Chang *et al.,* 1995) sequences. Generally, CTV dRNAs have been found to be composed of a simple fusion of the two termini of the genome, suggesting that internal specific noncontiguous segments are not required for interactions with the replicase complex. However, we found that internal contiguous sequences, which are probably not replication signals, influenced the accumulation of the dRNAs. While some 5′-termini were compatible with dRNA propagation, reducing the sizes of these termini, but keeping the minimal terminal sequence, decreased their accumulation levels (Fig. 1C). Some internal in-frame deletions did not abolish dRNA propagation (Fig. 2B), but caused a remarkable decrease in accumulation levels (results not shown). Also, insertion of the GFP ORF sequence in the junction site of a nonviable dRNA resulted in efficient replication (Fig. 6), suggesting that the spacing, rather than the specific nucleotide sequence of internal fragments, affected dRNA accumulation. One interpretation is that these sequences have interactions or form structures that contribute as spacers to hold the molecule in such a way that recognition signals for replication are appropriately exposed. Some deletions within these sequences could alter these interactions/structures, thereby causing interference with replication *in trans.* In some cases, further deletions restored the ability of the dRNA to be replicated, perhaps by reconstruction of compatible interactions/structures. Similar observations have been reported for other viruses. Deletion of the TMV sequence 3420–4902 resulted in the dRNA TMVΔRT that accumulated to low levels, yet additional progressive deletions resulted in variable changes in fitness (Lewandowski and Dawson, 1998). In TCV, replacement of deleted regions in nonviable DI RNAs with nonviral sequences of similar or greater lengths restored viability (Li and Simon, 1991).

Maintenance of a continuous ORF within many DI or dRNAs appears to be an essential requirement for their accumulation. Accumulation of CYMV dRNAs *in planta* depends on the maintenance of a long ORF resulting from fusion of ORF1 and ORF5 (White *et al.,* 1992). For efficient replication, RNA2-derived BBMV DI RNAs require the 5′ portion of the 2a ORF (Pogany *et al.,* 1997). In coronaviruses, the length of the ORF provides a competitive advantage for DI RNA accumulation (de Groot *et al.,* 1992; van der Most *et al.,* 1995). Sequence analyses of naturally occurring CTV dRNAs revealed that most had an ORF that continued to the recombination junction and some had an ORF that continued through the junction to the 3′-terminal sequence (Mawassi *et al.,* 1995a, b; Yang *et al.,* 1997a, b). Some dRNAs contain a second ORF (p23 ORF), although it is not clear whether this ORF is translatable. Three cDNA clones of dRNAs have been obtained from infected tissue that did not contain a significant 5′ ORF (Ayllón *et al.,* 1999). However, the fitness of the RNA transcripts of these clones is not known. Using synthetic dRNAs, our results clearly indicated that replication *in trans* requires maintenance of an ORF through most of the sequence. In general, the dRNAs with insertions, deletions, or mutations that preserved the reading frame were viable, whereas those that terminated the ORF were not viable.

It is unclear how translation of the ORF affects dRNA replication *in trans.* Translation may increase dRNA stability and its cytoplasmic half-life. In support for this explanation, the translatability of plant mRNAs has been shown to increase their accumulation (Vancanneyt *et al.,* 1990). Also, mRNAs that contain premature nonsense codons can be degraded by an mRNA decay pathway (Jacobson and Peltz, 1996). Even though translatability might protect CTV dRNAs from degradation, the majority of the dRNAs used in this study, including those with truncated ORFs, were relatively stable, with input RNA transcripts persisting in the protoplasts for more than 3 days.

Another possibility is that the dRNA translation product is needed for dRNA replication. The 126-kDa protein of TMV appears to be required *in cis* for accumulation of a class of TMV dRNAs (Lewandowski and Dawson, 2000). In contrast, several lines of evidence suggest that the CTV dRNA-encoded protein did not affect dRNA accumulation. The protein encoded from a viable dRNA did not act *in trans* to rescue nonviable dRNA. Replacement of a large internal CTV sequence (>60% of the ORF of CTVΔBstB:596) with the GFP ORF restored dRNA viability (Fig. 6), whereas premature termination of the ORF within the nonviral sequence destroyed viability. Furthermore, multiple frameshift mutations, which preserved *cis*-acting sequences but prevented production of the native protein, continued to be replicated (Fig. 7). The necessity for translation of the nonviral ORF (GFP) for accumulation of some dRNAs (Fig. 8) strengthens the argument that the translation process, rather than the encoded protein, affects dRNA accumulation. One explanation is that translation has a direct effect on the RNA structure, perhaps by unfolding the RNA to appropriately expose *cis*-acting signals.

CTV populations tend to be unusually complex with different amounts of unusually divergent genotypes and a range of dRNAs differing in size, abundance, and sequence. There is little information concerning how

these populations arise or what defines a stable population. However, the beginning of understanding factors that affect the fitness of dRNAs is a first step toward understanding CTV populations.

MATERIALS AND METHODS

dRNA construction and *in vitro* transcription

All constructs of dRNAs were derivatives of the full-length CTV cDNA clone pCTV9 (Satyanarayana *et al.,* 1999) and the dRNAs CTVΔBstB-ClaΔ456, CTVΔBstBΔ596, and CTVΔNco-ClaΔ368 described previously (Mawassi *et al.,* 2000). The sequence and nucleotide numbering are according to the CTV-T36 strain described by Karasev *et al.* (1995) (GenBank Accession Number U16304). Nucleotide numbering of each dRNA is shown in the diagrams depicted in the figures. The cDNA constructs were created using standard procedures described by Sambrook *et al.* (1989). When required, restriction endonuclease digestions, which created noncompatible ends, were followed by end-filling with the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA) prior to ligation. Nucleotide deletions or insertions and frameshift mutations were introduced by the polymerase chain reaction using primers with mutagenized sequences and Vent DNA polymerase (New England Biolabs). The enhanced GFP ORF (Crameri *et al.,* 1996) was used in some constructs. Plasmid DNA was prepared using the QIAGEN Plasmid Midi kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions.

In vitro transcription was carried out in a 25-μl reaction consisting of 2 μg *Not*I linearized plasmid DNA using SP6 RNA polymerase (Epicentre Technologies, Madison, WI) as described previously (Mawassi *et al.,* 2000). Freshly prepared *in vitro* transcripts were used directly for inoculation of protoplasts without further purification.

Protoplast isolation and inoculation

Protoplasts were isolated from fully expanded leaves of *N. benthamiana* (Navas-Castillo *et al.,* 1997) and transfection was carried out as described previously (Mawassi *et al.,* 2000). Transcripts of helper and dRNA were coinoculated into protoplasts with a mixture of 50 μl total volume. The inoculated protoplasts were incubated under fluorescent light at 26°C.

Analysis of viral RNA

Protoplasts were harvested and the total nucleic acids were extracted as described previously (Navas-Castillo *et al.,* 1997; Mawassi *et al.,* 2000). Agarose gel electrophoresis and Northern blot hybridization were performed as described by Lewandowski and Dawson (1998). Probing of the membrane with anti-digoxigenin–alkaline phosphatase Fab fragment and developing with CSPD chemiluminescent substrate were carried out according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). The 3′-terminal 900 nts of CTV-T36 cloned into pGEM-7Zf (Promega, Madison, WI) was used to synthesize positive- or negative-stranded RNA-specific probes using either SP6 or T7 RNA polymerase.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Drs. Shailaja Rabindran, Dennis J. Lewandowski, and Elisabeth Knapp as well as Ms. Cherie Sine for their comments on the manuscript. This research was supported in part by an endowment from the J. R. and Addie S. Graves family and grants from the Florida Citrus Production Research Advisory Board, U.S.D.A./A.R.S. Cooperative Agreement 58-6617-4-018, the U.S.–Israel Binational Agricultural Research and Development Fund, and the National Citrus Research Council. M. Mawassi was a recipient of Postdoctoral Award FI-0208-95 from the U.S.–Israel BARD. This work is part of the University of Florida Agricultural Experiment Station Journal Series R-07539.

REFERENCES

Ayllón, M. A., López, C., Navas-Castillo, J., Mawassi, M., Dawson, W. O., Guerri, J., Flores, R., and Moreno, P. (1999). New defective RNAs from citrus tristeza virus: Evidence for a replicase-driven template switching mechanism in their generation. *J. Gen. Virol.* **80,** 817–821.

Bar-Joseph, M., Marcus, R., and Lee, R. F. (1989). The continuous challenge of citrus tristeza virus control. *Annu. Rev. Phytopathol.* **27,** 291–316.

Bar-Joseph, M., Yang, G., Gafny, R., and Mawassi, M. (1997). Subgenomic RNAs: The possible building blocks for modular recombination of *Closteroviridae*. *Semin. Virol.* **8,** 113–119.

Chang, Y. C., Borja, M., Scholthof, H. B., Jackson, A. O., and Morris, T. J. (1995). Host effects and sequences essential for accumulation of defective interfering RNAs of cucumber necrosis and tomato bushy stunt tombusviruses. *Virology* **210,** 41–53.

Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. BioTechniques* **14,** 315–319.

de Groot, R. J., van der Most, R. G., and Spaan, W. J. (1992). The fitness of defective interfering murine coronavirus DI-a and its derivatives is decreased by nonsense and frameshift mutations. *J. Virol.* **66,** 5898–5905.

Febres, V. J., Ashoulin, L., Mawassi, M., Frank, A., Bar-Joseph, M., Manjunath, K. L., Lee, R. F., and Niblett, C. L. (1996). The p27 protein is present at one end of citrus tristeza virus particles. *Phytopathology* **86,** 1331–1335.

Hilf, M. E., Karasev, A. V., Pappu, H. R., Gumpf, D. J., Niblett, C. L., and Garnsey, S. M. (1995). Characterization of citrus tristeza virus subgenomic RNAs in infected tissue. *Virology* **208,** 576–582.

Holland, J. J., Kennedy, S. I. T., Semler, B. L., Jones, C. L., Roux, L., and Grabua, E. A. (1980). Defective interfering RNA viruses and the host-cell response. *Compr. Virol.* **16,** 137–192.

Holland, J. J. (1990). Defective viral genomes. *In* "Virology" (B. N. Fields and D. M. Knipe, Eds.), 2nd ed., Vol. 1, pp. 151–165. Raven Press, New York.

Jacobson, A., and Peltz, S. W. (1996). Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *Annu. Rev. Biochem.* **65,** 693–739.

Karasev, A. V. (2000). Genetic diversity and evolution of closteroviruses. *Annu. Rev. Phytopathol,* in press.

Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O. V., Hilf, M. E., Koonin, E. V., Niblett, C. L., Cline, K., Gumpf, D. J., Lee, R. F., Garnsey, S. M., Lewandowski, D. J., and Dawson, W. O. (1995). Complete sequence of the citrus tristeza virus RNA genome. *Virology* **208,** 511–520.

Knorr, D. A., Mullin, R. H., Hearne, P. Q., and Morris, T. J. (1991). *De novo* generation of defective interfering RNAs of tomato bushy stunt virus by high multiplicity passage. *Virology* **181,** 193–202.

Lewandowski, D. J., and Dawson, W. O. (1998). Deletion of internal sequences results in tobacco mosaic virus defective RNAs that accumulate to high levels without interfering with replication of the helper virus. *Virology* **251,** 427–437.

Lewandowski, D. J., and Dawson, W. O. (2000). Functions of the 126- and 183-kDa proteins of tobacco mosaic virus. *Virology* **271,** 90–98.

Li, X. H., and Simon, A. E. (1991). In vivo accumulation of a turnip crinkle virus defective interfering RNA is affected by alterations in size and sequence. *J. Virol.* **65,** 4582–4590.

Marsh, L. E., Pogue, G. P., Szybiak, U., Connell, J. P., and Hall, T. C. (1991). Non-replicating deletion mutants of brome mosaic virus RNA-2 interfere with viral replication. *J. Gen. Virol.* **72,** 2367–2374.

Mawassi, M., Karasev, A. V., Mietkiewska, E., Gafny, R., Lee, R. F., Dawson, W. O., and Bar-Joseph, M. (1995a). Defective RNA molecules associated with citrus tristeza virus. *Virology* **208,** 383–386.

Mawassi, M., Mietkiewska, E., Hilf, M. E., Ashoulin, L., Karasev, A. V., Gafny, R., Lee, R. F., Garnsey, S. M., Dawson, W. O., and Bar-Joseph, M. (1995b). Multiple species of defective RNAs in plants infected with citrus tristeza virus. *Virology* **214,** 264–268.

Mawassi, M., Satyanarayana, T., Gowda, S., Albiach-Martı, M. R., Robertson, C., and Dawson, W. O. (2000). Replication of heterologous combinations of helper and defective RNA of citrus tristeza virus. *Virology* **267,** 360–369.

Navas-Castillo, J., Albiach-Martı, M. R., Gowda, S., Hilf, M. E., Garnsey, S. M., and Dawson, W. O. (1997). Kinetics of accumulation of citrus tristeza virus RNAs. *Virology* **228,** 92–97.

Pappu, H. R., Karasev, A. V., Anderson, E. J., Pappu, S. S., Hilf, M. E., Febres, V. J., Eckloff, R. M. G., McCaffery, M., Boyko, V., Gowda, S., Dolja, V. V., Koonin, E. V., Gumpf, D. J., Cline, K. C., Garnsey, S. M., Dawson, W. O., Lee, R. F., and Niblett, C. L. (1994). Nucleotide sequence and organization of the eight 3′ open reading frames of the citrus tristeza closterovirus genome. *Virology* **199,** 35–46.

Pogany, J., Romero, J., and Bujarski, J. J. (1997). Effect of 5′- and 3′-terminal sequences, overall length, and coding capacity on the accumulation of defective RNAs associated with broad bean mottle bromovirus in planta. *Virology* **228,** 236–243.

Re, G. G., and Kingsbury, D. W. (1988). Paradoxical effects of Sendai virus DI RNA size on survival: Inefficient envelopment of small nucleocapsids. *Virology* **165,** 331–337.

Roux, L., Simon, A. E., and Holland, J. J. (1991). Effects of defective interfering viruses on virus replication and pathogenesis in vitro and in vivo. *Adv. Virus Res.* **40,** 181–212.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Satyanarayana, T., Gowda, S., Boyko, V. P., Albiach-Martı, M. R., Mawassi, M., Navas-Castillo, J., Karasev, A. V., Dolja, V., Hilf, M. E., Lewandowski, D. J., Moreno, P., Bar-Joseph, M., Garnsey, S. M., and Dawson, W. O. (1999). An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication. *Proc. Natl. Acad. Sci. USA* **96,** 7433–7438.

van der Most, R. G., Luytjes, W., Rutjes, S., and Spaan, W. J. (1995). Translation but not the encoded sequence is essential for the efficient propagation of the defective interfering RNAs of the coronavirus mouse hepatitis virus. *J. Virol.* **69,** 3744–3751.

Vancanneyt, G., Rosahl, S., and Willmitzer, L. (1990). Translatability of a plant-mRNA strongly influences its accumulation in transgenic plants. *Nucleic Acids Res.* **18,** 2917–2921.

White, K. A., Bancroft, J. B., and Mackie, G. A. (1992). Coding capacity determines *in vivo* accumulation of a defective RNA of clover yellow mosaic virus. *J. Virol.* **66,** 3069–3076.

Yang, G., Mawassi, M., Ashoulin, L., Gafny, R., Gaba, V., Gal-On, A., and Bar-Joseph, M. (1997a). A cDNA clone from a defective RNA of citrus tristeza virus is infective in the presence of the helper virus. *J. Gen. Virol.* **78,** 1765–1769.

Yang, G., Mawassi, M., Gofman, R., Gafny, R., and Bar-Joseph, M. (1997b). Involvement of a subgenomic mRNA in the generation of a variable population of defective citrus tristeza virus molecules. *J. Virol.* **71,** 9800–9802.

Zhang, C., and Simon, A. E. (1994). Effect of template size on accumulation of defective interfering RNAs in protoplasts. *J. Virol.* **68,** 8466–8469.